Formation and Breakdown of Citric Acid in Garcinia Fruit
(Xanthochymus guttiferae)

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Two hypotheses have been put forward to explain the large accumulation of organic acids in fruits: (a) organic acids are not formed in the fruit, but are translocated from the leaves to the fruits which would act merely as storage organs (1, 2); and (b) the presence of the organic acids in fruits is due to the conversion of carbohydrates to organic acids in the fruit vesicles and not to translocation from leaves (3, 4).

Recent studies in this laboratory have shown the presence in the tissues of Citrus aida of the enzyme system concerned with synthesis of citric acid (5) and suggested that organic acids may generally be formed in the fruit itself. To investigate this question further, the levels of several enzymes likely to be involved in the formation and breakdown were studied in the fruit tissues of Garcinia (Xanthochymus guttiferae) at different stages of growth.

An attempt was also made to purify three of these enzymes, namely, condensing enzyme, oxaloacetic carboxylase, and citric dehydratase. The data on the partial purification of the condensing enzyme have already been reported (6) and those on the purification of the other two enzymes are reported in this paper.

The results show that citric acid is formed from carbohydrate in Garcinia fruit tissue and suggest that its initial accumulation and subsequent disappearance are related to the changes in the activity of the condensing enzyme and other enzymes of the citric acid cycle.

EXPERIMENTAL PROCEDURE

Materials and Methods

Materials—The fruits, obtained from the University Botanical Garden, were collected during the same season. They were divided into groups of different sizes, each consisting of fruits of uniform size as determined equatorially by means of vernier calipers; the sizes chosen were 0.5, 1.5, 4.0, 4.5, 5.0 cm (unripe; green) and 5.0 cm (ripe; yellow), for enzyme studies.

The number of fruits used in an experiment varied inversely with the fruit size, from 100 in the case of the smallest fruits to 5 in that of the largest. Only fruits of the same size were used in a given experiment.

Preparation of Tissue Homogenate for Chemical Analysis—The fruits were peeled and seeded, and the mesocarpal tissue was finely sliced. A known weight of the tissue was immediately homogenized in a chilled mortar at 0” for 20 minutes, with half its weight of alumina (Alcoa, A 301, Aluminum Company of America) and twice the volume of 0.02 M potassium hydroxide when necessary. The extract was recovered by centrifugation at 18,400 × g and 0” for 30 minutes.

This extract was used directly for the assay of condensing enzyme, α-ketoglutaric oxidase, succinic dehydrogenase, fumarase, malic dehydrogenase, and oxaloacetic carboxylase and after dialysis for 8 hours, for the assay of citric dehydratase.

Preparation of Acetone Powder Extract—Freshly prepared fruits of the same size were peeled, seeded, and finely sliced in a cold room maintained at 0–4”. They were then weighed and blended with 10 volumes of cold (-10”) acetone for 1 minute in a Waring Blender. After filtration on a Buchner funnel with suction, the material was powdered by hand, spread over a large surface, and allowed to dry at room temperature. It was passed through a sieve of 50 mesh and stored in a vacuum desiccator at 4”.

The acetone powder was suspended in 10 volumes of 0.02 M potassium phosphate buffer, pH 7.0, stirred for 10 minutes at 0”, and the suspension centrifuged at 18,400 × g and 0” for 20 minutes. The supernatant solution was used for the assay of aconitase and isocitric dehydrogenase.

Methods—Citric acid was estimated according to the method of Saffran and Desmest (7), pyruvic acid by the method of Friedmann and Haugen (8), total sugar by the method of Hulme and Narain (9), and protein by the micro-Kjeldahl method.

The tricarboxylic acid cycle enzymes, excepting succinic dehydrogenase, were determined according to the methods described by Ramakrishnan (10) and Ramakrishnan and Martin (11), succinic dehydrogenase, by the method of Sreenivasamurthy and Swaminathan (12), oxaloacetic carboxylase, by that of Joshi and Ramakrishnan (13), and citric dehydratase, by that of Wheat and Ajl (14).

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A unit of condensing enzyme is defined as the amount, which, under the conditions of the assay, catalyzes the synthesis of 1 μmole of citrate in 40 minutes at 30”; specific activity is defined as units per mg of protein.

A unit of enzyme in the case of aconitase, isocitric dehydrogenase, fumarase, and malic dehydrogenase, is defined as the amount which causes a change in optical density of 0.001 per minute, at their respective wave lengths, and specific activity, as units per mg of protein.
A unit of enzyme in the case of α-ketoglutaric oxidase and pyruvic oxidase is defined as the amount which, under the conditions of the assay, causes a decrease of 0.001 in optical density at 600 μm per minute, and specific activity, as units per mg of protein.

A unit of succinic dehydrogenase enzyme activity is defined as the amount of enzyme which, under the conditions of the assay, causes the reduction of 1 μgram of triphenyltetrazolium chloride, and specific activity is as usual.

A unit of oxaloacetic carboxylase is defined as the amount of enzyme which, under the conditions of the assay, catalyzes the formation of 1 μmole of pyruvate at 30° in 30 minutes, and specific activity is as mentioned above.

In the case of citric desmolase, an enzyme activity is defined as the amount of enzyme which, under the conditions of the assay, catalyzes the dissimilation of 1 μmole of citrate at 37° in 30 minutes, and specific activity is as before.

To determine the reversible reaction catalyzed by oxaloacetic carboxylase, the enzyme synthesis of citrate from pyruvate was studied with the use of oxaloacetic carboxylase, condensing enzyme, and pyruvic oxidase. This complete system was used since Kalnitsky and Werkman (15) have suggested that the energy required for the formation of oxaloacetate may come from the pyruvic oxidase system.

The assay system contained phosphate buffer, pH 7.4, 20 μmole; neutralized cysteine hydrochloride, 10 μmole; coenzyme A, 12 units; diphosphopyridine nucleotide, 0.3 μmole; magnesium chloride, 8 μmole; adenosine triphosphate, 8 μmole; coenzyme A, 0.5 μmole; pyruvate, 40 μmole; oxaloacetic carboxylase preparation from Gaceinia fruit, Fraction C, 3 units; pyruvic oxidase from pigeon breast muscle (fraction obtained in the purification Step 3) (16), 40 μunits; condensing enzyme preparation from Gaceinia fruit, Fraction D, 10 units (6); and water to make the volume to 2.0 ml. The additions were made in test tubes kept in ice, after which the incubation was carried out at 30° for 1 hour; 0.5 ml of 25% trichloroacetic acid was then added, the protein was centrifuged, and citric acid and pyruvic acid were determined on the clear supernatant solutions.

Chemicals—Oxaloacetate was prepared by the method of Roberts (17), coenzyme A by that of Lipmann et al. (18), lithium acetyl phosphate by that of Avison (19), calcium phosphate gel as described by Keilin and Hartree (20), and alumina gel C’ by the method described by Colowick (21). Transacytelase in the form of an ammonium sulfate fraction was prepared from cell free extracts of Escherichia coli, N.R.C. 428, as described by Ramakrishnan and Martin (11). Other chemicals were commercial preparations.

Isolation of Oxaloacetic Carboxylase—Fifty milliliters of the cell-free extract of fruits of 4.0-cm diameter (Fraction A) were mixed with 10.5 g of solid ammonium sulfate and the mixture was allowed to stand at 0-4° for 10 minutes. The precipitate was removed by centrifugation at 12,800 X g and 0° for 15 minutes and dissolved in 17 ml of 0.02 M potassium phosphate buffer, pH 7.2 (Fraction B). To 17 ml of Fraction B was added 1.75 g of solid ammonium sulfate and the precipitate obtained was dissolved after centrifugation. To the supernatant fluid was added a further 1.75 g of solid ammonium sulfate and the mixture was allowed to stand at 0-4° for 15 minutes with occasional stirring. The precipitate collected by centrifugation at 4,600 X g and 0° for 15 minutes was dissolved in 7 ml of 0.02 M potassium phosphate buffer of pH 7.2 (Fraction C).

Seven milliliters of Fraction C were added to the residue obtained after centrifugation of 3.5 ml of calcium phosphate gel (8 mg of dry weight per ml); the mixture was stirred frequently for 10 minutes and centrifuged at 4,600 X g and 0° for 15 minutes. To the supernatant fluid (Fraction D) was added 0.5 volume of alumina gel C’ (0 mg of dry weight per ml), and the mixture was allowed to stand for 10 minutes with occasional stirring. The gel was recovered by centrifugation at 4,600 X g and 0° for 15 minutes, eluted with 7 ml of 0.1 M potassium phosphate buffer, pH 7.2, and the residue discarded after centrifugation. The supernatant solution (Fraction E) thus obtained showed the highest specific activity (Table I). The stability of the enzyme at -10° decreased with increasing purification; the crude extract remained active for 1 month but Fraction E remained active for only 2 days. The enzyme was destroyed by heating for 30 minutes at 45°.

Isolation of Citric Desmolase—Thirty milliliters of the cell-free extract of fruits of 5.0-cm diameter were dialyzed against 0.02 M potassium phosphate buffer, pH 7.0, for 8 hours in a cold room maintained at a temperature between 0-4°, to remove the citric acid present. The dialyzed extract was centrifuged in a Servall refrigerated centrifuge, at 287 X g and 0° for 10 minutes to remove denatured protein (Fraction A’).

Thirty milliliters of the dialyzed extract (Fraction A’) were mixed with 6.3 g of solid ammonium sulfate and the precipitated protein was discarded after centrifugation at 4,600 X g and 0° for 10 minutes. To the supernatant solution were added 4.2 g of solid ammonium sulfate and the mixture was allowed to stand at 0-2° for 15 minutes. The precipitate was removed by centrifugation at 4,900 X g and 0° for 15 minutes. The residue was dissolved in 0.02 M potassium phosphate buffer to a volume of 8 ml (Fraction B’).

Eight milliliters of Fraction B’ were then refractionated by the further addition of 0.28 g of solid ammonium sulfate and the residue was recovered after centrifugation at 4,600 X g and 0° for 15 minutes. The residue was dissolved in 0.02 M potassium phosphate buffer to a volume of 8 ml (Fraction C’).

Three milliliters of Fraction C’ were added to the residue obtained after centrifugation of 1.5 ml of calcium phosphate gel (8 ml of dry weight per ml). The mixture was stirred frequently for 10 minutes. The supernatant fluid (Fraction D’) thus obtained showed the highest specific activity (Table II). The stability of this enzyme is similar to that of oxaloacetic carboxylase.

RESULTS AND DISCUSSION

The results of the chemical analyses of fruits with reference to total sugar, citric acid, and protein are represented in Fig. 1. The total activity per fruit was estimated for each enzyme at the four stages of development. For this purpose, the average tissue protein per fruit was calculated by dividing the total tissue protein by the number of fruits used in the experiment. The results are presented in Table III.

The data in Fig. 1 show the reciprocal relation between change in acid and sugar content. This would indicate the transformation of sugar to citric acid in the fruit tissue itself due to the presence of the requisite enzyme systems. The decrease in citric acid in the ripe fruit suggests the possibility that this stage may perhaps be characterized by the presence of an enzyme system which effects the partial breakdown of citric acid.

It can be seen from Table III that the activities of the en-
zymes studied change differentially with development of the fruit, the most striking changes being with regard toaconitase which ceases to be active in the later stages, and citric desmolase, which is active only in the mature ripe fruits. Also, the condensing enzyme and malic dehydrogenase register an initial increase in the middle sized fruit followed by a decrease in the mature fruit, although not down to the original levels. Further, the total activities of pyruvic oxidase and oxaloacetic carboxylase are significantly greater in the middle sized and mature fruits than in the young fruit. Although the absence of aconitase activity suggests a block in the operation of the tricarboxylic acid cycle at the citrate level, the increase in the activity of the condensing enzyme suggests the formation of citrate at an accelerated pace, provided that a continuous supply of the precursors for citrate synthesis, particularly the C₄ precursor, can take place despite the block in the operation of the cycle. The possibility of such supply is indicated by the increased activity of oxaloacetic carboxylase. The appearance of a considerable amount of citric desmolase activity in the ripe fruit and the decrease in the citric acid content suggests the partial breakdown of citric acid by the activity of this enzyme. These studies show that changes in the activities of the tricarboxylic acid cycle and certain other enzymes during the development of the fruit can account adequately for the formation and accumulation of citric acid in the middle sized fruit and the decrease in the ripe fruit.

Studies carried out on the purification of citric desmolase and oxaloacetic carboxylase show that although citric desmolase isolated from Garcinia fruit resembles in its properties the enzyme detected in other tissues, oxaloacetic carboxylase seems to differ from that isolated from Micrococcus lysodeikticus (22) and pigeon liver (23) in not requiring a divalent ion for its activity. The observation that loss of oxaloacetic carboxylase activity due to dialysis for 8 hours against 0.02 M potassium phosphate can be restored to some extent by the addition of a supernatant solution from the boiled Garcinia fruit extract, but not by the addition of the ash of fruit extract, suggests that there may be some cofactor present in the tissue extract which is required for activity. Attempts made to find out the reversibility of the reaction catalyzed by means of the coupled system (Table IV) show that this enzyme may be similar to that isolated from M. lysodeikticus and pigeon liver, and that it plays a role in the formation of dicarboxylic acids. In the coupled reaction, the conditions are not optimal for all the enzymes, the pH of the assay system used being 7.0 with the result that the amount of citrate formed in the system is low. However, the formation of citrate in the coupled system, and the fact that omission of oxaloacetic carboxylase results in an absence of citrate synthesis shows the reversible nature of oxaloacetic carboxylase when coupled with a suitable system.

**SUMMARY**

A reciprocal relation between the change in citric acid and sugar content was observed during the development of Garcinia fruit. The activities of the tricarboxylic acid cycle enzymes

### Table I

**Partial purification of oxaloacetic carboxylase from fruit tissues of Garcinia**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume (ml)</th>
<th>Total protein (mg)</th>
<th>Specific activity (units/mg protein)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Cell-free extract</td>
<td>50</td>
<td>102</td>
<td>200.0</td>
<td>0.5</td>
</tr>
<tr>
<td>B. First (NH₄)₂SO₄ fraction</td>
<td>17</td>
<td>50</td>
<td>13.6</td>
<td>4.4</td>
</tr>
<tr>
<td>C. Second (NH₄)₂SO₄ fraction</td>
<td>7</td>
<td>35</td>
<td>3.5</td>
<td>10.1</td>
</tr>
<tr>
<td>D. Calcium gel supernatant</td>
<td>1</td>
<td>25</td>
<td>2.2</td>
<td>11.7</td>
</tr>
<tr>
<td>E. Alumina gel C₄ eluate</td>
<td>7</td>
<td>25</td>
<td>0.8</td>
<td>30.3</td>
</tr>
</tbody>
</table>

* 40 g of fresh weight.

### Table II

**Partial purification of citric desmolase from fruit tissues of Garcinia**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume of fraction (ml)</th>
<th>Total protein (mg)</th>
<th>Specific activity (units/mg protein)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Cell-free extract</td>
<td>30</td>
<td>141.0</td>
<td>0.8</td>
<td>100</td>
</tr>
<tr>
<td>A'. Cell-free extract</td>
<td>30</td>
<td>94</td>
<td>120.0</td>
<td>0.8</td>
</tr>
<tr>
<td>B. First (NH₄)₂SO₄ fraction</td>
<td>8</td>
<td>67</td>
<td>21.6</td>
<td>3.1</td>
</tr>
<tr>
<td>C. Second (NH₄)₂SO₄ fraction</td>
<td>3</td>
<td>62</td>
<td>4.5</td>
<td>13.8</td>
</tr>
<tr>
<td>D. Calcium gel supernatant</td>
<td>3</td>
<td>50</td>
<td>2.4</td>
<td>20.2</td>
</tr>
</tbody>
</table>

* 20 g of fresh weight.

![Fig. 1. Chemical composition of Garcinia at different stages of development.](http://www.jbc.org)
Oxaloacetic carboxylase effects the synthesis of citrate from pyruvate when coupled with pyruvic oxidase and condensing enzyme which suggests reversibility of the reaction catalyzed by oxaloacetic carboxylase.

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REFERENCES

### Table III

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Diameter of fruits</th>
<th>Enzymatic activity (expressed as enzyme units per fruit)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1.5 cm</td>
<td>4.0 cm</td>
</tr>
<tr>
<td>Condensing enzyme</td>
<td>38</td>
<td>315</td>
</tr>
<tr>
<td>Aconitase</td>
<td>740</td>
<td>0</td>
</tr>
<tr>
<td>Isochoric dehydrogenase</td>
<td>1050</td>
<td>1075</td>
</tr>
<tr>
<td>a-Retoglutaric oxidase</td>
<td>49</td>
<td>86</td>
</tr>
<tr>
<td>Succinic dehydrogenase</td>
<td>27</td>
<td>38</td>
</tr>
<tr>
<td>Fumarase</td>
<td>400</td>
<td>206</td>
</tr>
<tr>
<td>Malic dehydrogenase</td>
<td>810</td>
<td>3600</td>
</tr>
<tr>
<td>Oxaloacetic carboxylase</td>
<td>13</td>
<td>71</td>
</tr>
<tr>
<td>Pyruvic oxidase</td>
<td>98</td>
<td>141</td>
</tr>
<tr>
<td>Citric desmolase</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

### Table IV

<table>
<thead>
<tr>
<th>System</th>
<th>Citrate formed (µmole)</th>
<th>Pyruvate disappeared (µmole)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete system</td>
<td>1.10</td>
<td>3.0</td>
</tr>
<tr>
<td>Omit pyruvic oxidase</td>
<td>0.00</td>
<td>0.0</td>
</tr>
<tr>
<td>Omit pyruvate</td>
<td>0.00</td>
<td>0.0</td>
</tr>
<tr>
<td>Omit oxaloacetic carboxylase</td>
<td>0.08</td>
<td>2.0</td>
</tr>
<tr>
<td>Omit condensing enzyme</td>
<td>0.06</td>
<td>2.1</td>
</tr>
</tbody>
</table>

Change differentially with the development of the fruit, the most striking changes being with regard to aconitase which ceases to be active in the later stages, and citric desmolase which is active only in the mature fruits. Also, the condensing enzyme and malic dehydrogenase register an initial increase in the middle sized fruit followed by a decrease in the mature fruit, and the total activities of pyruvic oxidase and oxaloacetic carboxylase are significantly greater in the middle sized and mature fruits than in the young fruit.

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